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Invention:

The Use of β 1,3-Glucan-Containing Compositions to Potentiate Immune Responses by Upregulating the Expression of Costimulatory Molecules

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The Use of β 1,3-Glucan-Containing Compositions to Potentiate Immune Responses by Upregulating the Expression of Costimulatory Molecules

TECHNICAL FIELD

The present invention generally relates to the immunopharmacologic upregulation of a molecule of a family of B7 molecules to effectuate a costimulatory reaction that allows for an appropriate effector cell immune response.

BACKGROUND OF THE INVENTION

Glucan-containing compositions are polymers of glucose, and they are commonly found in the cell walls of bacteria, yeast, and various plant species. The glucans can be categorized according to the types of chemical linkages between their glucose monomers, and a common glucan-containing composition is β(1,3)-linked glucopyranose (commonly referred to as β glucan). Other common examples include mixtures of β-(1,3)-linked glucopyranose with β-(1,6)-linked glucopyranose. Glucans bearing these linkages have been shown to have immunopharmacological activity in animals and humans (DiLuzio, Trends Pharmacol. Sci. 1983, 4:344-7; U.S. Patent No. 05504079, 04/02/96; U.S. Patent No. 4900722, 02/13/90).

Although the prior art identifies both soluble and insoluble β-glucan-containing compositions from many microbial and plant sources as substances that can potentiate the immune response to a foreign material such as a microorganism or a tumor, the precise mode of action of these glucan-containing compositions has not been fully elucidated. Most of the prior art suggests that glucan-containing compositions potentiate immune responses by causing the activation of macrophages (DiLuzio, Trends Pharmacol. Sci., 1983, 4:344-347.). While

macrophage activation is certainly important for innate immunity through the enhanced destruction of pathogenic microorganisms and tumors, the macrophage is also the pivotal cell of the immune system for initiating adaptive immunity. In this role, the macrophage first engulfs foreign material in a process called phagocytosis, then processes these microbial proteins into peptides that are displayed on the macrophage cell surface in association with molecules of the major histocompatibility complex (MHC). Immune cells called T lymphocytes have clonally-derived receptors on their surfaces, and some of these receptors are invested with the ability to bind to a particular peptide so displayed. The end result is the initiation of an immune response involving humoral immunity (antibodies), cell-mediated immunity (killer cells), or both . There is evidence that beta glucan-containing compositions can potentiate both innate and adaptive immunity, but the exact mechanism for this enhancement is not known.

The macrophage and some other cell types have receptors in their surface membranes for β-glucan-containing compositions (Goldman, Exp. Cell Res., 1988, 174:481-90; Czop, J. Exp. Med., 1991, 173:1511-20). When β- glucan-containing compositions interact with the cell surface glucan receptor, the macrophage is activated and becomes capable of direct and indirect killing of the invading pathogen or tumor (Ross et al., Clin. Exp. Immunol., 1993, 92:181-4). However, macrophage activation alone is not responsible for the immunity enhancing effect of glucan. We have discovered a new mode of action of beta glucan-containing compositions that can explain its immunopotentiating effect, and this discovery is the subject of the present invention.

To understand the importance of this invention, one must understand the mechanism by which the immune response identifies a foreign substance (antigen), and develops a response that

can neutralize and/or eliminate the foreign substance before it causes significant morbidity or mortality. The immune system has evolved to discriminate between self and non-self (Miller et al., 1996, Curr. Opin. Immunol. 8:815-21). Non-self could be any of the myriad of potential pathogens for which humans and animals can be hosts, like viruses, bacteria, fungi, and parasites, or the non-self (or altered self) that is represented in the myriad of cancers that can arise from normal cells in an animal or human (Boon et al., Immunol. Today, 1997, 18:267-8). The challenge to the immune system is to identify that which is harmful non-self, and to mount a vigorous attack on this foreign material. Extraordinarily potent immune effector mechanisms have evolved, but these effector mechanisms have the potential of causing harm to the animal or human host if they become misdirected to harmless non-self (Goodnow, Proc. Natl. Acad. Sci. USA, 1996, 93:2264-71). Unfortunately, as good as the self/non-self discrimination is, the allergic and autoimmune diseases that animals and humans suffer result from errors in discrimination (Moller, Immunol. Rev., 1995, 144:1-314). It is important in the understanding of the present invention to know that the evolution of the immune system included means of avoiding, or at least minimizing, the errors in self/non-self discrimination.

To identify the extraordinary large array of foreign antigens that a human or animal can be exposed, the cells of the immune system evolved a large repertoire of clonally-derived receptors with the ability to bind specifically to the chemicals associated with foreign substances (antigens). To illustrate, we will describe the development of the repertoire of T lymphocyte receptors (TCR). A genetic recombination system has evolved whereby the TCR repertoire is created in the early development of T lymphocytes in the bone marrow (von Boehmer, Annu. Rev. Immunol., 1993, 6:309-26). The immature T cells migrate to the thymus where they

undergo a two step selection process that first eliminates all T cells bearing receptors that fail to recognize peptides presented on the surface of antigen presenting cells (APC, like macrophages, dendritic cells, and B cells) by major histocompatibility complex (MHC) molecules (Moller, Immunol. Rev., 1993, 135:5-242). Those that pass this test are then retested for strong reactivity against self peptides, thereby eliminating the vast majority of T cells that bear receptors capable of recognizing self peptides (Nossal, Cell, 1994, 76:229-39). The end result of this selection process is a pool of circulating T cells bearing receptors that, for the most part, will only recognize foreign antigens (central tolerance). Unfortunately, a small number of the T cells will bear receptors that can recognize certain self peptides (e.g., peptides from proteins not found in the thymus), and therefore have the potential of attacking certain self cells and causing immunopathology. To further protect against autoimmune responses, a mechanism known as peripheral tolerance has evolved (Hammerling et al., Immunol. Rev., 1999, 133:93-104). The understanding of peripheral tolerance is key to understanding the subject of the present invention.

If naive, but potentially autoreactive, T cells could bind to normal cells via their TCR and be induced to proliferate and differentiate into armed effector cells, these T cells could attack and kill self tissues causing disease and possibly death. To further protect against this potentiality, the immune system evolved a process whereby a naive T cell must receive **two signals** in order to proliferate and differentiate into an armed effector cell. The first signal is delivered by the TCR binding to its target peptide presented to it by an MHC molecule on the surface of an APC (Germain, Cell, 1994, 76:287-99). In the case of the T cell bearing a TCR that recognizes a self peptide, if the first signal was sufficient to allow it to become an armed effector cell, then interaction of that cell with normal tissue could result in the development of a clone of

autoreactive effector T cells that would attack the normal tissue. However, the mechanism of peripheral tolerance has evolved to prevent this from happening. A second signal (**costimulatory signal**) is required before the naive T cell can proliferate and differentiate into an armed effector cell, and that second signal is delivered by the interaction of CD28 molecules on the surface of the T lymphocyte with B7 molecules (B7-1 or CD80, and B7-2 or CD86) on the surface of an APC (Razi-Wolf et al., Proc. Natl. Acad. Sci. USA, 1992, 89:4210-14; Allison, Curr. Opin. Immunol. 1994, 6:414-9; June et al., Immunol. Today, 1994, 15:321-31; Lu et al., Curr. Opin. Immunol., 1997, 9:858-62). Indeed, if the first signal (TCR:MHC-peptide) is delivered in the absence of the second signal, not only does the T cell fail to become an armed effector cell, it actually goes into an inactive state called anergy (Powell et al., Immunol. Rev. 1998, 165:287-300). It is believed that such anergic cells eventually undergo a slow process of programmed cell death called apoptosis. Because most cells in an animal or human do not express, nor can they be induced to express, B7 molecules, these normal cells cannot serve as APC. Therefore, when a T cell bearing a TCR specific for a self peptide interacts with a normal tissue cell bearing that self peptide expressed in a cell surface MHC molecule, the T cell is made anergic, and is unable to become an effector cell capable of killing the normal cell. The combination of central and peripheral tolerance eliminates most of the potentially autoreactive T cells that arise in the course of T cell development.

It is the APC (macrophages, dendritic cells, B cells) that have the ability to express large amounts of B7 molecules on their surfaces. However, it is important for the understanding of the present invention to recognize that, for example, the macrophage does not normally express large amounts of B7 on its surface. It has been demonstrated that surface expression of B7 is

inducible, and when a macrophage interacts with certain microbial products (i.e., lipopolysaccharide or LPS), the gene coding for B7 is actively transcribed, and the macrophage begins to express B7 on its surface membrane (Foss et al., Infect. Immun., 1999, 67:5275-81). Only when the macrophage is activated in this way does it become an APC capable to providing both the first and second signal needed to cause specific T cells to proliferate and differentiate into armed effector cells.

To illustrate this further, let's take the example of the host immune response to an invading microorganism. The pathogen gains access to the blood or tissues of the host via one of several mechanisms, and begins to proliferate causing tissue damage. One of the first host defense cells to arrive on the scene is the macrophage, and the macrophage is capable of ingesting the microbes via the process of phagocytosis. Once the pathogen is ingested, it remains inside a vacuole that serves as a digestion chamber. Host cell enzymes are added to the chamber and the pathogen is killed and its proteins disrupted into peptides. The peptides are loaded onto newly formed MHC molecules (Germain, Cell 76:287-99), and then brought to the surface of the macrophage. The MHC:peptide complex is now ready to interact with a T cell bearing the correct TCR. At the same time, in order for the macrophage to be an effective APC, it must upregulate the expression of B7 genes, and begin to express large amounts of B7 costimulatory molecules on its surface. It is presently unclear just what microbial products are responsible for causing the upregulation of the B7 gene, and indeed, not all microorganisms ingested by macrophages cause the increased expression of B7 molecules (Gupta et al., Eur. J. Immunol., 1996, 26:563-70.). If sufficient B7 is expressed on the surface of the APC, the requisite T cells can be stimulated to become effector cells capable of killing the invading microorganisms.

It can be seen from this illustration of the function of the immune system that the B7 molecule and its surface membrane expression are key to the induction of the adaptive immune response. Failure of an APC to express B7 can actually result in the loss of protective T cells by the process of anergy. Should a T cell with an TCR specific for a peptide of an invading microorganism or tumor cell encounter that peptide on an APC that is not expressing B7, that T cell may be anergized and sent down the apoptotic pathway. This problem is addressed by the present invention.

We sought to identify a safe and effective pharmacologic agent that could upregulate the expression of the critical B7 costimulatory molecule on APC. If such an agent could be identified, it could be administered to an animal or human in a dosage sufficient to cause the cell surface expression of B7 on macrophages (or other APC), thereby allowing the macrophage to better serve the function of antigen presentation to T lymphocytes. It was known that macrophages had many receptors for highly conserved microbial constituents (Stahl, Curr. Opin. Immunol. 1992, 4:49-52; Hauschildt et al., Intl. J. Cytol.,1995, 161:263-70), and the interaction of the ligand on the microbe with its receptor on the macrophage has been shown, for example in the case of the LPS receptor, to cause the activation of the macrophage. It was known that macrophages also had glucan receptors, receptors into which β1,3-glucan-containing ligands would bind (Goldman, Exp. Cell Res., 1988, 174:481-90; Czop et al., J. Exp. Med., 1991, 173:1511-20). There was considerable evidence in the scientific literature that the β1,3-glucans could cause the activation of macrophages, making them more effective at phagocytosis and killing of microorganisms (DiLuzio, Trends Pharmacol. Sci., 1983, 4:344-347). The β1,3-glucans have been administered to animals and humans for years with no untoward effects, so

we wondered whether this class of pharmacologic agents would upregulate the cell surface expression of B7 molecules on macrophages. We performed a laboratory experiment in which macrophages taken from the peritoneal cavity of donor mice, or tumor macrophages were incubated *in vitro* with various amounts of β1,3-glucan-containing compositions (see below). By using fluorescent-labelled anti-B7 antibodies, we discovered that whereas both the peritoneal macrophages and the tumor macrophages did not express large amounts of cell surface B7 molecules before the addition of β1,3-glucan-containing compositions, after incubation with the β1,3-glucan-containing composition these cells began to express large amounts of B7 molecules on their surface membranes. Our invention, therefore, involves the identification of β1,3-glucan-containing compositions as pharmacologic agents capable of causing macrophages to express surface membrane B7 molecules. Our invention includes the administration of such β1,3-glucan-containing compositions to animals and humans to increase the effectiveness of APC in providing the critical second signal necessary for inducing specific T lymphocytes to proliferate and differentiate into armed effector cells capable of protecting against harmful non-self, like pathogenic microorganisms and cancer.

Although the B7 gene has been cloned and methods described for its expression in various cell types (see U.S. Patent No. 6,071,716, 06/06/00; U.S Patent No. No.6,077,833, 06/20/00), our invention is distinguished from this prior art in that we do not need to clone the B7 gene and manipulate its expression in a host cell in order to augment an immune response. Moreover, although there have been descriptions made of methods of blocking the activity of B7 and its required interaction with CD28 (for example see U.S. Patent No. 6,090,914, 07/18/00; U.S. Patent 5,869,050, 02/09/00), we are not aware of any prior art that provides for a simple

immunopharmacological upregulation of the expression B7 molecules to enhance immune responses to foreign antigens in animals or humans.

SUMMARY OF THE INVENTION

This invention relates to a method of augmenting the immune response to a foreign antigen (i.e., pathogenic microorganism, tumor) by the use of β1,3-glucan-containing compositions. The invention involves the administration of the β1,3-glucan-containing composition to an animal or human in a dose and by a route that serves to bring a critical amount of this material to the vicinity of macrophages or monocytes (blood, tissues, secondary lymphoid organs). The β1,3-glucan-containing composition interacts with a specific glucan receptor on the surface of these APC, initiating an intracellular signal that results in the upregulation and surface expression of a family of molecules called B7 (i.e., B7.1 and B7.2). The B7 molecules are costimulatory molecules that are critical in the provision of a second signal to specific T lymphocytes that have received a first signal through a specific cell surface receptor (TCR) that interacts with a foreign peptide in the context of an MHC molecule on the surface of the APC. When the naive T lymphocyte has received both signals, it proliferates and then differentiates into an armed effector T lymphocyte that can effectuate one of the important defense mechanisms of the immune response (i.e., cell mediated immunity or antibody-mediated immunity). Because APC like macrophages do not normally express much cell surface B7, they are not effective in delivering the all critical second signal to T lymphocytes. Our discovery that β1,3-glucan-containing compositions can cause APC like macrophages to express large amounts of B7 on their surfaces, allows us to provide a pharmacological intervention to make the APC more

effective in initiating the adaptive immune response, and therefore, more effective in providing protection against foreign antigens like microbes and tumors.

It is therefore, one object of this invention to provide a method for improving the induction of adaptive immunity in humans and animals. It is a further object of this invention to provide an immunopharmacological composition that, when administered to a human or animal, will enhance the immune response to foreign antigens such as antigens in vaccines, pathogenic microorganisms, and tumor cells.

BRIEF DESCRIPTION OF DRAWINGS AND PHOTOGRAPHS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

For a further understanding of the nature and objects of the present invention, reference should be had to the following detailed description, taken in conjunction with the accompanying drawings and photographs, in which like elements are given the same or analogous reference numbers and wherein:

Fig. 1 is an immunofluorescence photomicrograph demonstrating the upregulation and cell surface expression of B7.2 on the mouse tumor macrophage cell line P388D1 treated *in vitro* with a β-1,3-glucan-containing composition.

Fig. 2 is an immunofluorescence photomicrograph demonstrating the upregulation and cell surface expression of B7.2 on mouse peritoneal macrophages treated *in vitro* with a β-1,3-glucan-containing composition.

Fig. 3 is an immunofluorescence photomicrograph demonstrating the upregulation and cell surface expression of B7.2 on mouse peritoneal macrophages treated *in vivo* with a β-1,3-glucan-containing composition.

GENERAL DESCRIPTION AND PREFERRED MODE FOR CARRYING OUT THE INVENTION

The examples below illustrate the preferred mode of carrying out the invention. In these examples, we demonstrate that a β 1,3-glucan-containing composition can cause the upregulation of cell surface expression of B7 molecules on macrophages. We also demonstrate that the same β 1,3-glucan-containing composition can activate macrophages and enhance their rate of bacterial phagocytosis. We further demonstrate the enhancement of an immune response to a foreign antigen (sheep red blood cells) in mice given the β 1,3-glucan-containing composition orally.

For these experiments we used a preparation of β 1,3-glucan from the common Baker's yeast *Saccharomyces cerevisiae* (Bacon et al., Biochem. J., 1969,114:557-566; Fleet et al., J. Gen Micro., 1976, 94:180-92; Williams et al. Carbohydrate Res., 1991 219:203-13; U.S. Patent No. 05032401, 07/16/91; U.S. Patent No. 05504079, 04/02/96; U.S. Patent No. 05849720, 12/15/98), but another source of β 1,3-glucan could easily be substituted as the scientific and patent literature is replete with examples of immunopotentiating compositions of β 1,3-glucan from other yeasts, bacteria, and plants. We used a common method of preparing an alkali extract of the yeast, which was then subjected to sonication in order to disrupt the larger glucan globules into smaller particles in the size range of 1 micron. However, our invention is meant to encompass any β 1,3-glucan-containing composition, ranging from whole Baker's yeast to purified soluble β 1,3,-glucan, that upon interaction with a macrophage causes the upregulation and cell surface expression of B7 molecules.

EXAMPLES

Example 1. Mouse tumor macrophage cell line (P388D1) treated *in vitro* with a β 1,3-glucan-containing composition: upregulation of B7 surface membrane expression.

In this experiment the tumor macrophage-like cell line P388D1 (ATCC, Manassas, VA.) was grown in wells of eight-chambered microscope slides containing tissue culture media with 10% fetal bovine serum at 37°C in 5% CO_2. Cells were stimulated with media (control cells) or media containing 100 μg/ml β 1,3-glucan-containing composition for 1 hour. During this time, it was demonstrated (data not shown) that the macrophages ingested large quantities of the β 1,3-glucan-containing particles. After incubation, the stimulant was washed away and replaced with growth media and incubated as before. Approximately 24 hours post-stimulation, live cells were incubated with a fluoresceinated antibody directed to mouse B7.2 (BD PharMingen, San Diego, CA). After an incubation of 1 hour, the unbound antibody was washed away and the cells were examined under a Nikon Eclipse 400 fluorescence microscope. Upon examination of more than 50 cells, it was concluded that the glucan treatment had the effect of increasing the amount of surface membrane B7.2 from negligible before (Figure 1A) to substantial after treatment (Figure 1B). Note the bright yellow halo around the macrophages in the glucan-treated cells. From this experiment, it is concluded that a β 1,3-glucan-containing composition can cause the upregulation of a B7 costimulatory molecule in this particular mouse macrophage-like tumor cell line..

Example 2. Mouse peritoneal macrophages treated *in vitro* with a β 1,3-glucan-containing composition: upregulation of B7 surface membrane expression.

In this example of the upregulation and surface expression of the B7.2 costimulatory molecule on mouse macrophages by a β 1,3-glucan-containing composition, mouse peritoneal macrophagse were harvested by peritoneal lavage with cold tissue culture medium. The cells were placed in tissue culture medium containing 10% fetal bovine serum and placed in the wells of eight-chambered microscope slides and incubated at 37°C and 5% CO_2 for two hours. The non-adherent cells were removed by washing in warm medium, and the macrophages were found to attach to the glass substrate of the microscope slide. Cells were stimulated with media (control cells) or media containing 100 μg/ml β 1,3-glucan-containing composition for 1 hour. During this time, it was demonstrated (data not shown) that the macrophages ingested large quantities of the β 1,3-glucan-containing particles. After incubation, the stimulant was washed away and replaced with growth media and incubated as before. Approximately 24 hours post-stimulation, live cells were incubated with a phycoerythrin-conjugated antibody directed to mouse B7.2. After an incubation of 1 hour, the unbound antibody was washed away and the cells were examined under a Nikon Eclipse 400 fluorescence microscope. Upon examination of more than 50 cells, it was concluded that the glucan treatment had the effect of increasing the amount of surface membrane B7.2 from negligible before (Figure 2A) to substantial after treatment (Figure 2B). Once again, note the bright yellow hallo around the glucan-treated cells. From this experiment, it is concluded that a β 1,3-glucan-containing composition can cause the upregulation of the B7 costimulatory molecule on mouse peritoneal macrophages *in vitro*.

Example 3. Mouse peritoneal macrophages treated *in vivo* with a β 1,3-glucan-containing composition: upregulation of B7 surface membrane expression.

In this experiment, mice were injected via the intraperitoneal route with 100 μg of a β 1,3-glucan-containing composition in 0.25 ml of sterile saline or with 0.25 ml sterile saline alone. After 24 hours, peritoneal macrophages were harvested and processed for immunofluorescence as described in Example 2 using a phycoerythrin-conjugated antibody toward mouse B7.2. Upon examination of more than 50 cells, it was concluded that the glucan treatment had the effect of increasing the amount of surface membrane B7.2 from negligible before (Figure 3A) to substantial after treatment (Figure 3B). From this experiment, it is concluded that a β 1,3-glucan-containing composition administered to a mouse by the intraperitoneal route can cause the upregulation of the B7 costimulatory molecule on mouse peritoneal macrophages.

Example 4. Mouse peritoneal macrophages stimulated *in vivo* with a β 1,3-glucan-containing composition: enhanced phagocytosis of bacteria.

In this experiment mice were fed 132 μg/kg of a β 1,3-glucan-containing composition daily for 18 days. Peritoneal macrophages were harvested by peritoneal lavage as described in Example 2. Peritoneal macrophages were plated into each well of a 8-well chambered slide. Macrophages were allowed to adhere for approximately 4 hours followed by washing 3X with PBS to remove non-adherent cells. 100 μl of fluoroscein isothiocyanate-labeled bacteria (bioparticles, 1:20 dilution in PBS) was added to each well and incubated for 20 minutes at 37°C. Following incubation, the slides were rinsed in PBS to remove excess bio-particles. Bio-particles remaining on the outside of the macrophages were quenched by the addition of 100 μl of a 50 μg/ml ethidium bromide solution to each well and incubated for 10 minutes at room temperature in the dark and then analyzed by fluorescence microscopy. The results are shown in Table 1.

It was concluded from this experiment that a β 1,3-glucan-containing composition can activate macrophages for the enhanced engulfment of microorganisms.

Table 1.

	Cells w/o particles	Cells with particles	Ave# particles/cell**
No Glucan	31.8 (64.9%)	17.2 (35.1%)	2.2
Glucan	18.7 (36.0%)	33.3 (64.0%)	3.4

Example 5. Mouse peritoneal macrophages stimulated *in vivo* with a β 1,3-glucan-containing composition: potentiation of immune responses.

This experiment was done in two parts. Mice were fed 132 μg/kg of a β 1,3-glucan-containing composition daily for 19 days. On day 2 and day 8, mice were given an injection of 1 x 10^9 sheep red blood cells (SRBC) via the intraperitonel route. For the detection of serum anti-SRBC antibodies, blood was drawn from the mice eleven days after the last injection and allowed to coagulate. After centrifugation the serum component was aspirated and used for hemagglutination assays. Serum was serially diluted from 1: to 1:20,480 in PBS in a 96-well microtiter plate. An equal amount of 1.5% washed SRBC was added to each serum dilution, mixed well and incubated at 4°C for 6 hours. The reciprocal of the final dilution causing noticeable agglutination of the SRBC was defined as the titer. The results are shown in Table 2.

Table 2.

Treatment	Reciprocal Titer
No glucan	1841.1
Glucan	2906.7

It was concluded from this experiment that the administration of oral glucan can potentiate the primary IgM antibody response to a T cell dependent antigen (SRBC).

In the second part of the experiment mice, spleens were harvested aseptically from the same mice eleven days after the last injection of SRBC. The tissue was dissociated by mincing followed by sieving through a 210 μm polypropylene mesh into growth medium. After lysis of red blood cells the lymphocytes were counted and diluted to 1 x 10^8 cells/ml for plaque-forming cell assays to determine the number of IgM secreting B cells. A 1:100 dilution of spleen cells was mixed with a 7.5% SRBC solution, placed in a Cunningham-Szenberg chamber and incubated at 37°C for two hours . After incubation the plaques were enumerated. The results are shown in Table 3.

Table 3.

Treatment	**IgM Plaques**
0 mg Glucan	175 ± 50
10 mg Glucan	625 ±275

It was concluded from this experiment that the administration of oral glucan can increase the number of B lymphocytes making antibodies to SRBC, another example of the immunopotentiating ability of β 1,3 glucan.

The preceding examples are not intended limit the scope of Applicant's invention, but serve as an explanatory tool in the many advantages of Applicant's invention. Further, although one general embodiment of Applicants' invention has been described in the preceding Description, it will be understood by those skilled in the art that various embodiments fit within the scope of the invention.